# Bacterial mechanosensitive ion channels

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# Chapter 2

## Activating Mechanosensitive Channels from *Mycobacterium tuberculosis* and *Lactococcus lactis* in the absence of tension

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**Abstract**

Mechanosensation is one of the principal mechanisms by which mechanosensitive (MS) ion channels control the movement of molecules in and out of the cell. MS channels are prevalent in all kingdoms of life and play a crucial role in sensing diverse stimuli such as touch, hearing, gravity and changes in osmolarity. Ongoing efforts to elucidate the gating mechanisms of MS channels are based mainly on functional studies of the mechanosensitive channel from *Escherichia coli* (Ec-MscL), taking advantage of the crystal structure of a homologous protein from *Mycobacterium tuberculosis* (Tb-MscL). Experimental work on Tb-MscL itself has been limited due to the high tension required for activating this channel. Here, we report on the activation of Tb-MscL by hydrophobic gating in the absence of applied membrane tension. We compare the properties of Tb-MscL with those of Ec-MscL and a closely related homolog Ll-MscL from *Lactococcus lactis*. We found that the $20^{th}$ and $21^{st}$ positions on both Tb-MscL and Ll-MscL, and additionally the $22^{nd}$ and $23^{rd}$ residues of Ll-MscL cause spontaneous opening of the channel when chemically modified with a positively charged moiety. However, the $15^{th}$, $22^{nd}$ and $23^{rd}$ positions in Tb-MscL do not lead to spontaneous channel opening when modified with the positively charged moiety. We also show that this sensitivity to the gating signal depends on the lipid environment. Our results emphasize structural differences around the hydrophobic gate region of the Tb-MscL and Ll-MscL, and the importance of lipid bilayer composition on the functioning of MscL channels in general.

## Introduction

The mechanosensitive channel of large conductance (MscL) from *Escherichia coli* is one of best-studied mechanosensitive (MS) ion channels (1, 2). MS channels in the cytoplasmic membrane maintain the cell integrity when they are confronted with a severe osmotic downshift (3-5). Under these conditions, water influx into the cell increases the turgor pressure, thereby generating severe tension in the membrane. MscL channels sense the tension in the membrane and open a large, transient, non-selective pore through which solutes and water are released.

Four years after the discovery of the *mscL* gene (6), and following numerous functional studies on *E. coli* MscL (Ec-MscL), a crystal structure of MscL was obtained from the homologue isolated from *Mycobacterium tuberculosis* (Tb-MscL)(7). The "closed" or "nearly closed" Tb-MscL structure at 3.5 Å resolution revealed that the channel is made up of five identical subunits. The polypeptides have a short cytoplasmic N-terminus (S1), followed by two transmembrane segments (TM1 and TM2) that are linked by a periplasmic loop (PL), and a C-terminal domain that protrudes into the cytoplasm (Figure 1). However, the crystal structure was not sufficient to reveal the gating mechanism of MscL. Functional studies on Tb-MscL were complicated by the very high tension necessary for its opening (8). Therefore, functional studies continued mainly on Ec-MscL, and the results were rationalized by employing the Tb-MscL crystal structure, with the assumption that both channels share a common molecular mechanism for detecting and responding to membrane tension (9). Electrophysiology and mutagenesis studies suggest that Tb- and Ec-MscL are structurally and functionally similar. However, some well-studied Ec-MscL gain-of-function (GOF) mutations do not translate to Tb-MscL GOF mutants. For instance, mutants of Val-23 in Ec-MscL, corresponding to Val-21 in Tb-MscL, behave differently (11). While the substitution of Val-23 for smaller or polar residues yielded a very severe GOF phenotype in *E. coli* (11), mutating Tb-MscL Val-21 into an alanine or arginine generated a much milder GOF phenotype (8).

Another striking difference between the two channels relates to position Gly-22 in Ec-MscL, which corresponds to Ala-20 in Tb-MscL (8, 10). Gly-22 in Ec-MscL is critical for channel gating. Substitution of glycine with hydrophilic amino acids or chemical compounds dramatically affects the channel gating (11, 12). While hydrophilic amino acids generate a GOF phenotype *in vivo* (12), chemical modification of G22C in Ec-MscL via a cysteine-specific positively charged compound [2-(trimethylammonium) ethyl]methanethiosulphonate bromide, MTSET, resulted in channels that could be activated even in the absence of applied tension (13). On the contrary, Tb-MscL, substituted at Ala-20 with charged amino acids, functioned just like the wild-type protein (10).

Both Ala-20 and Val-21 in Tb-MscL are located within the so-called "hydrophobic lock", that is, the Leu-17xxxVal-21 region on TM1 helix; x =alanine or valine or glycine. In Ec-MscL, 6 of the 8 very severe GOF mutants, generated by substitutions to either a more hydrophilic or a charged amino acid, are in the analogous region Leu-19xxxVal-23 (11). These observations formed the basis for the proposal of "hydrophobic gating" (14) as the gating mechanism of MscL (15). According to this mechanism, the hydrophobicity of the pore constriction acts as a functional block of the gate. However, when the solvent environment of the hydrophobic residues changes, the gate opens and the channel begins to conduct ions. This hypothesis found support from molecular dynamics simulations (16), and flying-patch patch clamp studies (17); it is also supported by experimental work on heteropentameric Ec-MscL channels (18).
MscL from *Lactococcus lactis* (Ll-MscL) with the highest sequence identity in its pore region to both Tb- and Ec-MscL has been recently characterized (19). As in Ec-MscL, an increase in the hydrophilicity at the position of Gly-20 in Ll-MscL resulted in a GOF phenotype (19). In the light of these findings, it is surprising that hydrophobic gate mutants in Tb-MscL at the $20^{th}$ and $21^{th}$ positions showed either a slight GOF behavior or functioned normally (8, 10). Some researchers have suggested that intrinsic properties of Tb-MscL, such as the lipid environment and/or other modulators that might be present in the native environment, could cause the unexpected behavior of Tb-MscL (8).

Here, we aim at testing the hydrophobic gating mechanism of Tb-MscL and Ll-MscL. We generated cysteine mutants in the hydrophobic pore region of both channels and modified the individual thiol groups with MTSET. This was done with the aim to break the hydrophobic lock by chemically attaching a positively charged moiety in the pore. We studied the modified protein in *E. coli* and *L. lactis*, and in proteoliposomes. Unlike the previous unexpected findings

on Tb-MscL, where mutating A20 to some hydrophilic residues did not give GOF mutants (10), we found that the hydrophilicity of the 20$^{th}$ and 21$^{st}$ position play an important role in the channel gating. Interestingly, the threshold tension for gating depends on the lipid composition of the membrane bilayer as shown by a comparison of channel function in two different bacterial hosts. These results indicate that the lipid environment should be taken into account in analyzing MscL channel function from when using the Tb crystal structure as a model. Furthermore, even though, in Tb-MscL, changes at positions 22 and 23 had no influence on the channel gating, attaching a charge via MTSET at the corresponding residues in Ll-MscL affected the gating. This highlights the structural differences that exist around the hydrophobic gate region of these homologous proteins. The finding that destabilization of the hydrophobic pore activates Tb-MscL, hence eliminating the need for applying tension to the lipid bilayer, offers new possibilities for functional and structural studies on Tb-MscL (18). Finally, identifying particular amino acids that can be used for remote activation of Tb- and Ll-MscL offers new, alternative channels for delivery and sensory applications.

## Materials and Methods

### Design of synthetic genes and cloning

The MscL amino acid sequence from *L. lactis* (gi 12725155) and *M. tuberculosis* (gi 6016604) were used to produce the corresponding codon-optimized genes Ll-*mscL* and Tb-*mscL* respectively, using the programme DNA 2. The sequences were codon-optimized for expression in *E. coli* and *L. lactis,* and were finally synthesized by Geneart, Germany. The *Nco*I and *Spe*I restriction sites were added to the N- and C-terminus of the sequence, respectively for cloning. The four genes were amplified by PCR from the Geneart plasmid using the following forward (F) and reverse (R) primers:

**Table 1.** Primers for amplification of synthetic genes

| Primer type | Primer sequence |
|---|---|
| | Ll-*mscL* optimized for *E. coli*: |
| Forward | 5'GGCCAGTTAATTAAGAGGTACCAGC |
| Reverse | 5'GGCCGTCAAGGCCTAGGCGCG |
| | Ll-*mscL* optimized for *L. lactis* |
| Forward | 5'GGGCGAATTGAAGGAAGGCCG |
| Reverse | 5'GCGGGCAGTGAAAGGAAGGC |
| | Tb-*mscL* optimized for *E. coli* |
| Forward | 5'GGCCAGTTAATTAAGAGGTACCAGC |
| Reverse | 5'GGAAGGCCGTCAAGGCCTAGGC |
| | Tb-*mscL* optimized for *L. lactis* |
| Forward | 5'CTCACTATAGGGCGAATTGGCG |
| Reverse | 5'GTGAGCGGAAGGCCCATGAGGC |

After amplification, the respective genes, previously cut with *Fat*I and *Spe*I were inserted into p1BAD vector that was cut with *Nco*I and *Spe*I(18).

**Mutagenesis**

The cysteine mutants were created by QuikChange mutagenesis, using the following primers (only forward primers are given).

**Table 2.** Ll-*mscL* codon-optimized for *E. coli*

| Mutation | Primer (forward)sequence |
|---|---|
| L15C | 5'CTGCGTGGCAATGTTTGTGATCTGGCAGTTGGTGTTATTATTGG |
| G20C | 5'CTGGATCTGGCAGTTTGTGTTATTATTGGTGCAGCATTTACCGC |
| V21C | 5'CTGGATCTGGCAGTTGGTTGTATTATTGGTGCAGCATTTACCGC |
| I22C | 5'CTGGATCTGGCAGTTGGTGTTTGTATTGGTGCAGCATTTACCGC |
| I23C | 5'CTGGATCTGGCAGTTGGTGTTATTTGTGGTGCAGCATTTACCGC |

**Table 3.** Ll-*mscL* codon-optimized for *L. lactis*

| Mutation | Primer (forward)sequence |
|---|---|
| L15C | 5'CGTGGAAATGTTTGTGATCTTGCTGTTGGAGTTATTATTGGAGC |
| G20C | 5'CTTGATCTTGCTGTTTGTGTTATTATTGGAGCTGCTTTT ACAGC |
| V21C | 5'CTTGATCTTGCTGTTGGATGTATTATTGGAGCTGCT TTTACAGC |
| I22C | 5'CTTGATCTTGCTGTTGGAGTTTGTATTGGAGCTGCTTTTACAGC |
| I23C | 5'CTTGATCTTGCTGTTGGAGTTATTTGTGGAGCTGCTTTTACAGC |

**Table 4.** Tb-*mscL* codon-optimized for *E. coli*

| Mutation | Primer (forward) sequence |
|---|---|
| V15C | 5'GCACGTGGCAATATTTGTGATCTGGCAGTTGCAGTTGTTATTGGC |
| A20C | 5'GTTGATCTGGCAGTTTGTGTTGTTATTGGCACCGCATTTACCGC |
| V21C | 5'GTTGATCTGGCAGTTGCATGTGTTATTGGCACCGCATTTACCGC |
| V22C | 5'GTTGATCTGGCAGTTGCAGTTTGTATTGGCACCGCATTTACCGC |
| I23C | 5'GTTGATCTGGCAGTTGCAGTTGTTTGTGGCACCGCATTTACCGC |

**Table 5**. Tb-*mscL* codon-optimized for *L. lactis*

| Mutation | Primer (forward) sequence |
|---|---|
| V15C | 5'GCTCGTGGAAATATTTGTGATCTTGCTGTTGCTGTTGTTATTGG |
| A20C | 5'GTTGATCTTGCTGTTTGTGTTGTTATTGGAACAGCTTTTACAGC |
| V21C | 5'GTTGATCTTGCTGTTGCTTGTGTTATTGGAACAGCTTTTACAGC |
| V22C | 5'GTTGATCTTGCTGTTGCTGTTTGTATTGGAACAGCTTTTACAGC |
| I23C | 5'GTTGATCTTGCTGTTGCTGTTGTTTGTGGAACAGCTTTTACAGC |

**Bacterial growth**

The growth of the cysteine mutants was investigated in small cultures (50 ml) without aeration for *L. lactis* and with vigorous aeration for *E. coli*. For *L. lactis* NZ9000, M17 media supplemented with 0.5% v/v glucose and 5µg/ml chloramphenicol was used; cells were grown at 30 $^{o}$C. The cells were induced by nisin A, using a 1 to 1000 dilution of the culture supernatant from the nisin A-producing *L. lactis* NZ9700 strain (39) at $A_{600}$ of about 0.6. The control culture was not induced.

*E. coli* PB104 was grown in LB medium supplemented with ampicillin (100 µg/ml) and chloramphenicol(10 µg/ml). The cultures were incubated at 37 $^{o}$C with shaking at 200 rpm. At $A_{600}$ of 0.6, one culture was induced with 0.1% (v/v) arabinose and the control culture did not receive arabinose; in both cases 0.4% (v/v) glycerol was added as an alternative carbon source.

**Cell viability**

The *E. coli* cells were grown in LB media supplemented with 0.5M NaCl at 37$^{o}$C with continuous shaking at 200 rpm until $A_{600}$ of about 0.25. The cells were then induced by adding 0.1% arabinose (v/v) and grown for one hour to express the *mscL* gene. After another hour of growth, the cells were diluted 1 to 20 into fresh media having the following osmotic conditions: osmostatic (LB plus 0.5M NaCl), osmotic downshift (LB only), osmostatic with 1 mM MTSET and osmotic downshift with 1 mM MTSET. The cells were grown for a further 15 minutes, after which serial dilutions were made. Plating was done in triplicate on LB agar plates, which were later incubated at 37$^{o}$C overnight.

**Membrane vesicle preparation**

Membrane vesicles from *E. coli* PB104 (ΔmscL: $Cm^{res}$ recA ) (40), carrying plasmid p1BAD with a particular gene, were prepared as described in (18 and19). The cells were grown in 2L pH- and $pO_2$-controlled bioreactors at 37 $^{o}$C. MscL expression was initiated by the addition of 0.1% (v/v) arabinose. Cells were harvested by centrifugation and washed using 25 mM Tris-HCl pH8. The harvested cells were lysed using a cell disrupter (Type TS/40; Constant Systems) at 25,000 psi. The resulting cellular debris was removed by centrifugation at 18,460 x g for 30 min at 4°C. The supernatant was then centrifuged at 145,400 x g for 90 min at 4°C. The resulting vesicles were homogenized in 25mM Tris-HCl, pH8 to a final concentration of 0.7g/ml and subsequently frozen in liquid nitrogen and stored at -80°C until needed.

**Protein isolation**

Protein was isolated as described before (25). Briefly, membrane vesicles corresponding to 2.8 g wet weight were solubilized in detergent-buffer (1% v/v Triton X-100, 300 mM NaCl, 50 mM sodium phosphate, 35 mM imidazole, pH 8). The unsolubilized material was removed by centrifugation at 26,7000 x g for 20 min at 4°C. The supernatant was added to detergent-buffer equilibrated Ni-NTA matrix and incubated at 4°C for 30 min with gentle mixing. The unbound protein was rinsed off the matrix with wash buffer (0.2% v/v Triton X-100, 50 mM sodium phosphate, 300 mM NaCl, 35 mM imidazole). A second wash buffer (50 mM histidine 0.1% v/v Triton X-100, 50 mM sodium phosphate, 300 mM NaCl, pH 8) was used to elute nonspecific bound proteins. MscL was eluted from the Ni-NTA

column using an elution buffer (235 mM histidine, 0.1% v/v Triton X-100, 300 mM NaCl, 50 mM sodium phosphate pH 8).

**Protein reconstitution into preformed liposomes**

The protein reconstitution into artificial liposomes was based on Koçer *et al*., (25). Briefly, azolectin was dissolved in buffer A (10 mM sodium phosphate and 15 0mM NaCl, pH 8) at 20 mg/ml. The dissolved lipid was subsequently subjected to five freeze - thaw cycles, using liquid nitrogen and 50$^{o}$C water bath, respectively. Then, the liposomes were extruded eleven times through a 400 nm polycarbonate filter. A portion of the extruded lipid vesicles (250 µl) was mixed with 20 µl of 10% Triton X-100 and incubated for 5 min at 50°C. After incubation, 300 µl of purified MscL protein (0.2- 0.25 mg/ml) was added to the mixture of lipid vesicles and detergent, and incubated for 30 min at 50$^{\circ}$C. One volume (570 µl) calcein fluorescent dye (200 mM, pH 8) was added to the mixture together with 200 mg of Biobeads$^{TM}$. The tubes were wrapped in aluminum foil and incubated overnight at 4°C with gentle mixing. The proteoliposomes were separated from free calcein by using a Sephadex G50 size-exclusion column.

**Fluorescence dequenching assay**

The assay was performed as described before (25). Briefly, 2 µl of proteoliposomes were diluted into 2.2 ml buffer (10mM sodium phosphate, 150 mM NaCl and 10 mM EDTA, pH 8). After 1 min, MscL in proteoliposomes were activated either by adding MTSET (1 mM) or lyso-phosphatidylcholine (LPC; 4.5 µM). The fluorescence was measured continuously at 510 nm for 12 minutes. The total fluorescence of the sample was determined by dissolving the proteoliposomes by the addition of 0.5% (v/v) Triton X-100 after 10 min of recording.
The % calcein release from the liposomes through the channel was calculated using the formular:

% Calcein release = ((b-a)/ (m-a))*100

a = initial or background flurescence
b = fluorescence at a particular time (t)
m = maximum fluorescence after Triton X-100 treatment

The values were used to plot the release, i.e. % calcein release vs time.

**Site-directed PEGylation**

Site-directed PEGylation was performed as described previously (18). Briefly, the following were the treatments (i) control proteoliposomes with no MTSET and no MTS-PEG 500, (ii) proteoliposomes incubated first with MTSET and later incubated with 1.5 mM of MTS-PEG 5000 for 5 min, (iii) proteoliposomes incubated only with1.5 mM of MTS-PEG 5000 for 5 min. Subsequently, the protein mixture was separated on 15% SDS-PAGE gel.

**Preparation of giant spheroplasts**

Giant spheroplasts from *E. coli* (PB104) were prepared as described previously (2, 40). Briefly, an overnight culture from a single colony was used to inoculate fresh LB medium, supplemented with ampicillin (100 µg/ml) and chloramphenicol (10 µg/ml). At $A_{600}$ of about 0.4, a 1 to 10 dilution was made into fresh LB medium with cephalexin (60 µg/ml). The cells were grown for 1 hour and *mscL* expression was initiated by addition of arabinose (0.1% v/v) and glycerol (0.4% v/v). The cells were harvested by centrifugation in a swing-out rotor at 4,000 x g at 4$^{\circ}$C for 10 min. The cell wall was partially digested by adding the following reagents sequentially: 120 µl of 1M Tris-HCl buffer pH 7.8, 120 µl of Lysozyme (5mg/ml), 30 µl of DNAse (5mg/ml) and 120 µl of 0.125 M EDTA pH 8. The digestion was allowed to proceed for 1.5 to 3 minutes and stopped by adding a stop solution (20 mM $MgCl_2$, 10 mM Tris-HCL pH

7.8, 0.7 M sucrose). The spheroplasts were harvested in a sucrose cushion by low speed centrifugation at 4,000 x g at 4°C for 1-5 min, and they were aliquoted and stored at -20°C.

### Patch Clamp

An aliquot of 2-5 µl of the spheroplast sample was placed in the bath containing the following buffer: 200 mM KCl, 90 mM $MgCl_2$, 10 mM $CaCl_2$, 5 mM HEPES-KOH, pH 7. Calibrated 100-µL pipettes (Drummond Scientific) with a 1-µm tip diameter were pulled using the Sutter Instrument P-1000. The pipette tip was filled with the same buffer as the bath. All recordings were performed with excised patches under the same conditions (-20 mV, gain 10, sampling rate of 30 µs). The data were amplified and filtered at 10 kHz using an Axopatch 1D amplifier, sampled at 33 kHz in a Digidata 1322A digitizer and analyzed with pCLAMP10 software (Molecular Devices).

## Results

### Ll-MscL cysteine mutants are more sensitive to the lipid environment than Tb-MscL mutants when expressed in *E. coli* and *L. lactis*

As a first step to test the hydrophobic gating hypothesis, we changed the hydrophobic residues between 20 and 23 of Tb-MscL (A20, V21, V22, I23) and Ll-MscL (G20, V21, I22, I23), individually into cysteines to enable subsequent chemical modification of the pore constriction by MTSET (Fig.1). Additionally, we changed a residue (position 15) further down the pore region to a cysteine (V15 in Tb-MscL and L15 in Ll-MscL), which, in a molecular dynamics simulation, was reported to be more sensitive to tension than the wild type Tb-Mscl (20). We cloned both the wild type (WT) and cysteine mutants of Ll-MscL and Tb-MscL in *E. coli* PB104 (ΔmscL: $Cm^{res}$ recA) and in *L. lactis* NZ9000 by using p1BAD and pNZ8048 plasmids, respectively. The resulting mutants were studied both *in vivo* and *in vitro* in the presence and absence of MTSET.

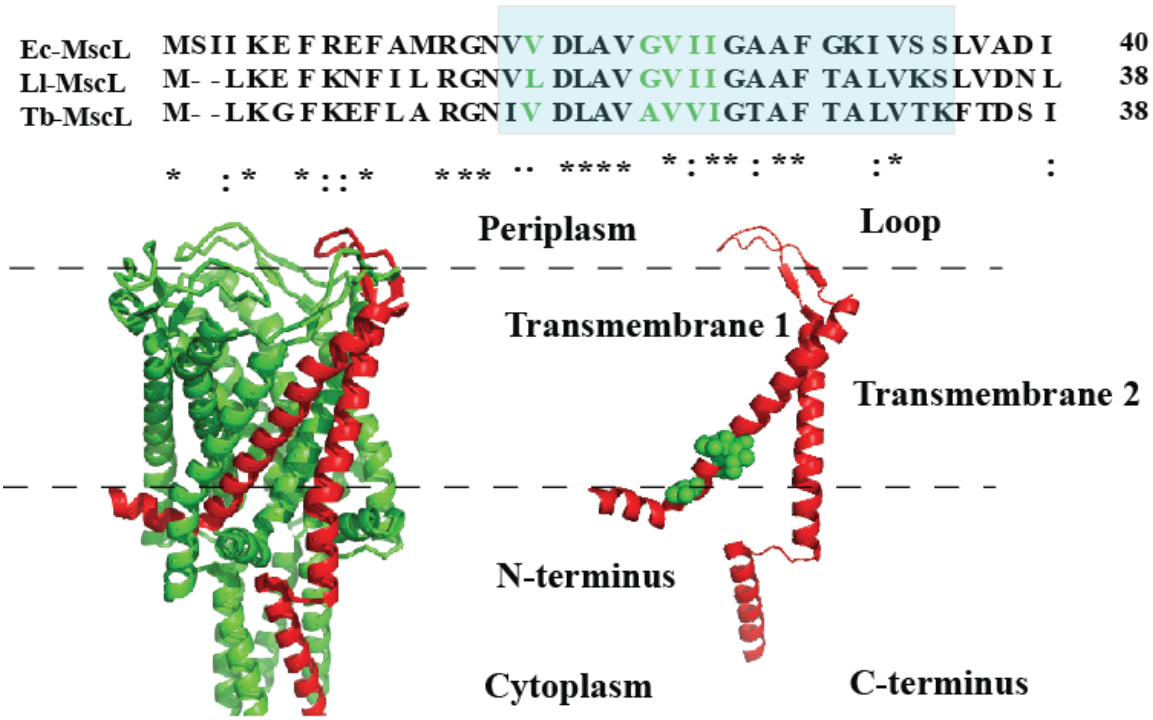

**Figure 1.** Tb-MscL crystal structure and part of the sequence alignment of three MscL homologues. On the left is shown the Tb-MscL homopentamer with one subunit highlighted in red. On the right is depicted a single subunit with residues mutated shown as green spheres. The top shows part of a multiple sequence alignment of MscL from *E. coli* (Ec-MscL), *L. lactis* (Ll-MscL) and *M. tuberculosis* (Tb-MscL). The area highlighted in light blue is the TM1 domain and colored green are the residues mutated and analyzed in this study. The homology among the proteins is indicated as follows (*) fully conserved residues (:) conservative substitutions.

Next, to check for possible effects of individual cysteine mutations on the functioning of MscL channels in *E. coli* and *L. lactis* cells, we followed the growth behavior of both organisms with or without induction of Tb-*mscL* or Ll-*mscL*. Surprisingly, Tb-MscL cysteine mutants and WT expressed in *L. lactis* caused a 50% reduction in overall growth yield based on A600 values (Fig. 2A). Lowering the expression level by reducing the inducer concentration up to 20 times did not remedy the reduction in growth. As a control, we transformed *L. lactis* cells with an empty vector (pNZ8048 without any *mscL* gene) and the cells grew normally (Fig. 2A). The observed reduction is not influenced by the cysteine mutation since both all the three mutants and WT showed same level of reduction. When we expressed Tb-MscL cysteine mutants and WT in *E. coli* host, there was no reduction in the growth (Fig. 2B). Together, these results suggest that heterologous expression of Tb-MscL in *L. lactis* affects growth but the expression of the protein has no influence on *E. coli* cells.

When Ll-MscL cysteine mutants and WT were expressed in *L. lactis* and *E. coli*, only $G20C^{LlMscL}$ and $I22C^{LlMscL}$ pore mutants showed about 50% reduction in growth (Fig. 2C and 2D). The cysteine mutation at these two positions affects the proper functioning of the channel, hence the observed reduced growth yield. With the Ll*mscL* gene the effect of the cysteine mutation is evident in the two hosts as shown by the reduced viability of $G20C^{LlMscL}$ and $I22C^{LlMscL}$. In the case of Tb-MscL gene, the corresponding cysteine mutations do not have an effect. Interestingly, the $LlMscL^{WT}$ expressed in *L. lactis* did not show reduction in growth as was the case with $Tb\text{-}Mscl^{WT}$. This may suggest that the Ll-MscL cysteine mutants are more sensitive to the lipid environment than Tb-MscL mutants when expressed in *E. coli* and *L. lactis*. However, despite this reduction in growth for Tb-Mscl cysteine mutants and WT when expressed in *L. lactis* cells, significant amounts of Tb-MscL were produced (Fig. 2E). Characteristic growth curves of *E. coli* cells expressing $G20C^{LlMscL}$ and $LlMscL^{WT}$ are presented in Fig. 2F, and they show that reduction in growth occurs when inducer is added to the medium.

Overall, the results suggest that Tb-MscL imposes different growth constraints on *E. coli* and *L. lactis*. All tested cysteine mutants from Tb-MscL grew as good as the WT Tb-MscL. On the other hand, cysteine mutants of Ll-MscL (G20C and I22C) presented a gain of function behavior relative to the WT Ll-MscL, which is in accordance with previous reports on *E. coli* MscL ($G20C^{LlMscL}$ and $I22C^{LlMscL}$) expressed in both *E. coli* and *L. lactis* (22).

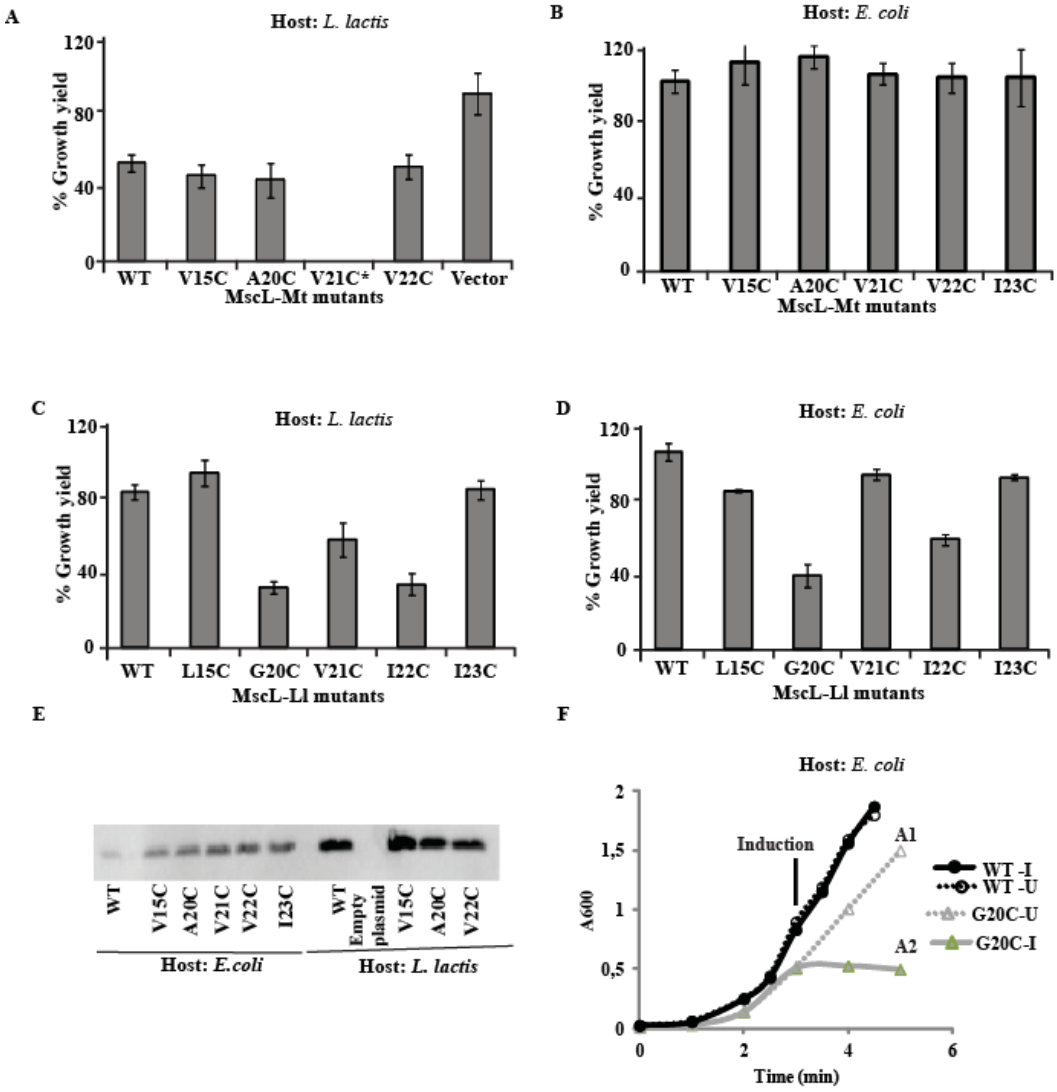


**Figure 2.** Effect of MscL cysteine mutants on the growth of *E. coli* and *L. lactis* cells. (A) Top left, Tb-MscL cysteine mutants and WT expressed *L. lactis* showed about 50% reduction in growth. (B) Top right, Tb-Mscl cysteine mutants and WT showed normal growth after MscL expression in *E. coli*. (C and D) Middle panel, Ll-MscL G20C and I22C show marked reduction in growth when protein expression is initiated in both *E. coli* and *L. lactis.* The reduction in growth of L15C, V21C and I23C expressed in *E. coli*, and V21C expressed in *L. lactis,* was moderate and the reduction in growth yield was at most 20%. L15C and I23C affect growth less when expressed in *L. lactis* compared to *E. coli*. (E) Bottom left, the SDS-PAA gel shows the protein expression levels of Tb-MscL cysteine mutants expressed in *E. coli* and *L. lactis.* (F) Bottom right, typical growth curves showing normal growth (LlMscL WT, induced-I and uninduced-U) and affected growth (G20C induced-I vs G20C uninduced). The growth yield % was calculated by diving the A600 value of the cells with and without induction.

### Tb-MscL and Ll-MscL can be triggered to open *in vivo* under osmostatic conditions

After studying the growth behavior of *E. coli* and *L. lactis* upon expression of Tb- and Ll-MscL, we next tested if the channels gate in the absence of applied tension. To do that, we explored the effect of increased hydrophilicity of the pore region by covalently attaching a positively charged group to cysteine residues using MTSET. The channel-gating characteristic was tested in a viability assay. In these experiments, the channels were kept in their closed form under osmostatic conditions or forced-open upon osmotic downshift. In control experiments, MTSET was excluded. We assessed the accessibility of the pore residues to the MTSET as described previously (23). Briefly, after an initial growth in hyperosmotic media (1086 mOsm) (24), the cells were either transferred to a medium of the same (osmostatic) or lower osmolarity (osmotic downshift, 248 mOsM) with or without 5 mM MTSET. If the pore region is accessible to MTSET, we expect to see the channel opening in the absence of tension, hence a reduction in cell growth due to leakage of cellular components, already at osmostatic conditions. However, if the pore region becomes accessible to MTSET upon opening, we expect a reduced cell growth after the osmotic downshock. Once the cells

were transferred to the osmostatic or downshock media, serial dilutions of each sample were made, and cells were cultivated on LB agar plates. The resulting colony-forming units were counted and expressed as a percentage of each osmostatic (mock) condition.

In *L. lactis*, Tb-MscL mutants V15C$^{\text{Tb-Mscl}}$ and A20C$^{\text{Tb-Mscl}}$ could not be opened by MTSET, neither in the osmostatic nor osmotic downshift conditions, as can be seen from the similar viability of the cells under these conditions, suggesting that these residues are not accessible to MTSET in the closed and open form of the channel. V22C$^{\text{Tb-Mscl}}$ showed a slightly lower viability only when the cells were transferred from high to low osmolality medium in the presence of MTSET (Fig. 3A). Mutants V21C$^{\text{Tb-Mscl}}$ and I23C$^{\text{Tb-Mscl}}$ could not be made in time due to primer annealing problems and were left out.

In *E. coli*, A20C$^{\text{Tb-Mscl}}$ mutant could be activated by MTSET in the absence of tension as can be judged from a severe reduction in viability (Fig. 3B). The V21C$^{\text{Tb-Mscl}}$ mutant could also be opened by MTSET, both under osmostatic and osmotic downshift conditions. Of the other mutants, only V15C$^{\text{Tb-Mscl}}$ showed some reduction in viability only upon osmotic downshift (Fig. 3B), suggesting that the corresponding Cys residues are not accessable in the channel's closed state and only partially accessable in the open state.

In the case of *L. lactis* MscL pore mutants expressed in *L. lactis*, G20C$^{\text{LlMscL}}$, V21C$^{\text{LlMscL}}$ and I22C$^{\text{LlMscL}}$ could be opened upon treatment with MTSET even under osmostatic conditions (Fig. 3C), indicating that these pore residues are accessible to MTSET in their closed form. These mutants behaved similar in *E. coli* (Fig. 3D). The I23C $^{\text{LlMscL}}$ mutant was affected by MTSET under both osmostatic and downshift conditions in *L. lactis*; the viability dropped significantly. When I23C $^{\text{LlMscL}}$ was expressed in the *E. coli* host, it showed reduction in viability, under osmotic downshift conditions. Together, the data on I23C $^{\text{LlMscL}}$ suggest that the accessibility of this position depends on the environment in which the protein is expressed.

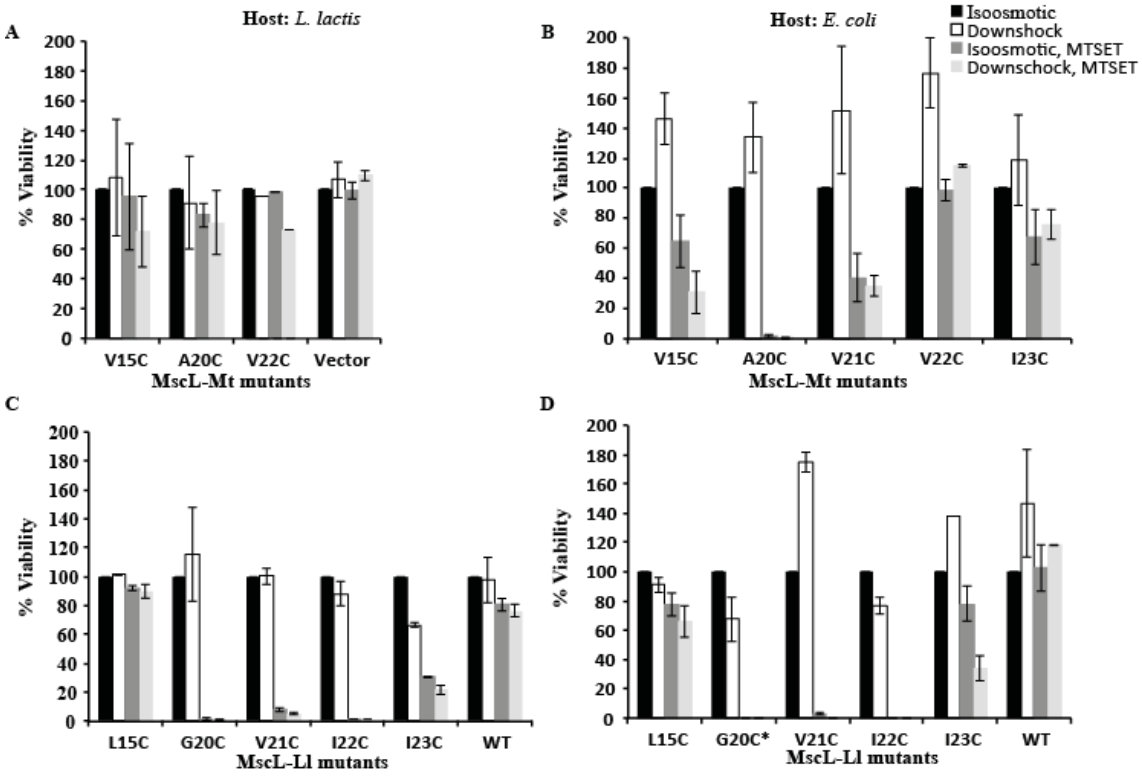

**Figure 3.** *In vivo* activation of MscL via chemical modification of cysteine residues. Left and right panels indicate *L. lactis* and *E. coli*, respectively. Tb- and Ll-MscL cysteine mutants were tested for their activation by MTSET under osmostatic and osmotic downshift conditions. Cells were grown in LB media (*E. coli*) and M17 plus glucose (*L. lactis*). After *mscL* expression was induced, the cells were challenged for 15 min under four different osmotic conditions: osmostatic (black bars), osmotic downshift (white bars), osmostatic plus MTSET (dark gray bars), and osmotic downshift/MTSET (light gray bars), as specified in Materials and Methods. Cells from serial dilutions were plated on LB agar and colony-forming units were calculated. The number of colonies was expressed as the percent over the mock shock condition. (A and B), Tb-MscL mutants expressed in *L. lactis* and *E. coli,* respectively; (C and D), Ll-MscL mutants expressed in *L. lactis* and *E. coli*, respectively.

### Tb-MscL and Ll-MscL can be activated *in vitro* in the absence of applied membrane tension

To further elucidate the tension-independent activation of MscL channels from *L. lactis* and *M. tuberculosis*, we purified these proteins, reconstituted them in synthetic liposomes, and followed their activation using a fluorescence-dequenching assay (25). To this end, wild-type MscL and cysteine mutants were incorporated into liposomes prepared from azolectin at a 1 to 50 protein to lipid ratio (wt:wt). During the reconstitution process, a self-quenching fluorescent dye was encapsulated into the liposomes. In the assay, the MscL opening was triggered *in situ* by the addition of MTSET to the osmostatic bulk solution. If MTSET activates MscL channels, these channels open and release the fluorescent dye. The dequenching of fluorescent dye upon its release from the liposomes into the bulk solution generates a fluorescence signal at 515 nm.

Unlike the host-dependent activity of Tb-MscL channels *in vivo*, their reconstituted activity of the protein was not dependent on the host they were isolated from. Tb-MscL mutants, $A20C^{Tb\text{-}Mscl}$ and $V21C^{Tb\text{-}Mscl}$, which were not accessible to MTSET in *L. lactis* but were accessible in *E coli*, could be activated with MTSET when reconstituted in liposomes (Figure 4A). The $A20C^{Tb\text{-}Mscl}$ and $V21C^{Tb\text{-}Mscl}$ released the calcein with first order rate constants of 2.31 ± 0.25 and 2.45 ± 0.08, respectively (Table 6). On the other hand, the activity of $I23C^{Tb\text{-}Mscl}$ was minimal. $V15C^{Tb\text{-}Mscl}$ and $V22C^{Tb\text{-}Mscl}$ did not show any activity. The possible reasons for the lack of activity for $V22C^{Tb\text{-}Mscl}$ and $I23C^{Tb\text{-}Mscl}$ MscL could be either inability of MTSET to access these particular amino acid positions or inability of the added charges to gate the channel.

**Table 6.** First order rate constants for calcein release from proteoliposomes

| MscL type | Cysteine mutant | Rate constant $k$ $min^{-1}$ + S.D | |
|---|---|---|---|
| | | **MTSET** | **LPC** |
| | V15C | **ND** | 1.88 ± 0.03 |
| | A20C | 2.31 ± 0.25 | 1.77 ± 0.19 |
| **TbMscL** | V21C | 2.45 ± 0.08 | 1.45 ± 0.12 |
| | V22C | **ND** | 1.38 ± 0.11 |
| | I23C | **ND** | 1.99 ± 0.06 |
| | | | |
| | L15C | **ND** | 0.46 ± 0.02 |
| **LlMscL** | G20C | 5.34 ± 1.42 | 1.81 ± 0.18 |
| | V21C | 1.84 ± 0.34 | 1.47 ± 0.03 |
| | I22C | 0.52 ± 0.05 | 0.56 ± 0.18 |
| | I23C | 0.41 ± 0.03 | 0.38 ± 0.09 |

ND not determined since mutants not responsive to MTSET

In the case of $G20C^{LlMscL}$, $V21C^{LlMscL}$, $I22C^{LlMscL}$ and $I23C^{LlMscL}$ these can be opened by MTSET (Figure 4C). $G20C^{LlMscL}$ and $V21C^{LlMscL}$ show faster kinetics (rate constants of 5.34 ± 1.42 and 1.84 ± 0.34, respectively) and higher calcein release than $I22C^{LlMscL}$ and $I23C^{LlMscL}$. $L15C^{LlMscL}$ is the only cysteine mutant of LlMscL that did not respond to MTSET activation. All

channels derived from LlMscL were activated by LPC too (Figure 4D). However, G20C$^{LlMscL}$ and V21C$^{LlMscL}$ show faster kinetics compared to L15C$^{LlMscL}$, I22C$^{LlMscL}$ and I23C$^{LlMscL}$. This trend was also observed when using MTSET as a trigger for activation.

In order to discriminate between these two possibilities, i.e. Inability of MTSET to access these particular amino acid positions or inability of the added charges to gate the channel, we first tested the accessibility of the cysteines to MTSET. For that, we incubated the proteoliposome samples first with MTSET. After 15 min, we removed the free MTSET and dissociated the MscL channels by incubating the proteoliposomes with SDS buffer without β-mercaptoethanol. We added a cysteine-specific pegylating compound, methoxypoly(ethylenglycol)5000 amidopropionyl methanethiosulfonate (MTS-PEG5000), to the mixture and monitored a possible shift in the migration of the protein, using SDS-polyacrylamide gel electrophoresis. If a cysteine is labeled by MTSET it cannot react with MTS-PEG5000, and we would not see a shift on SDS-PAGE. If, on the contrary, the cysteine does not react with MTSET, subsequent treatment with MTS-PEG5000 will rise to a molecular weight shift on SDS-PAGE. Our data indicate that V22C$^{Tb\text{-}MscI}$ and I23C$^{Tb\text{-}MscI}$ channels do not interact with MTS-PEG5000 (Fig. 4E line b of each mutant). However, if they were not pre-incubated by MTSET, the channels reacted with MTS-PEG5000 (Fig. 4E line c of each mutant). The results of MTS-PEG5000 labelling of V22C$^{Tb\text{-}MscI}$ and I23C$^{Tb\text{-}MscI}$ show a similar shift in the band (Fig. 4E) as the controls A20C$^{Tb\text{-}MscI}$, V21C$^{Tb\text{-}MscI}$ and G22C$^{Ec\text{-}MscI}$. Thus, V22C$^{Tb\text{-}MscI}$ and I23C$^{Tb\text{-}MscI}$ are accessible to both MTSET and MTS- PEG5000 just like the controls A20C$^{Tb\text{-}MscI}$, V21C$^{Tb\text{-}MscI}$ and G22C$^{Ec\text{-}MscI}$.

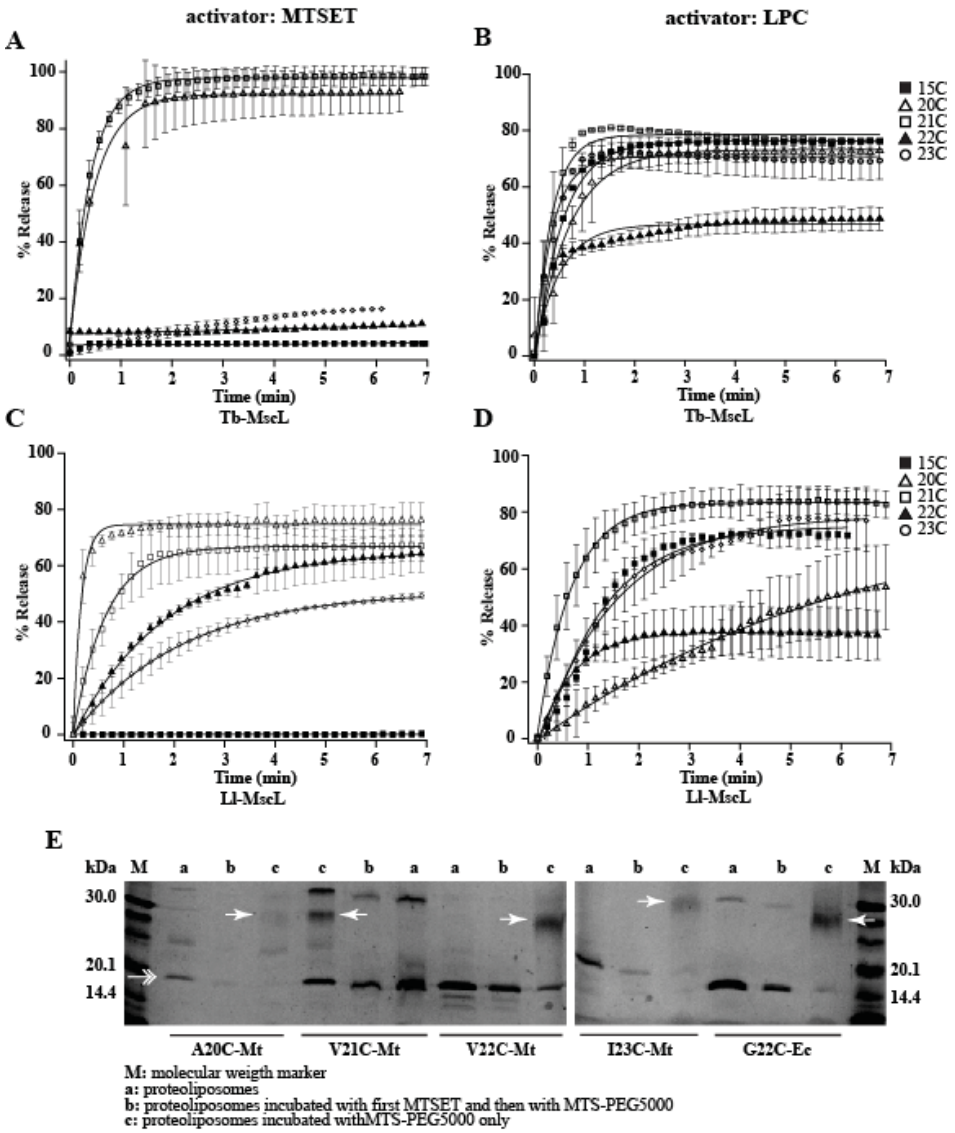


**Figure 4.** *In vitro* activation of MscL. In (A-D), the left and right panels indicate the effect of MTSET and lysophosphatidylcholine (LPC) on channel activity, respectively. MscL mutants in proteoliposomes were tested for their activity

under osmostatic conditions, using the fluorescence-dequenching assay. (A) Tb-MscL mutants activated with 1 mM MTSET; (B) Tb-MscL mutants activated with 4.5 μM lysophosphatidylcholine; (C) Ll-MscL mutants activated with 1 mM MTSET; (D) Ll-MscL mutants activated with 4.5 μM lysophosphatidylcholine; (E) PEGylation of free cysteine residues of Tb-MscL cysteine mutants in proteoliposomes with methoxypoly(ethylenglycol)5000 amidopropionyl methanethiosulfonate (MTS-PEG5000). M: molecular weigth marker, Line a: the control proteoliposomes with no MTSET and no MTS-PEG 500, Line b: proteoliposomes first incubated with 1 mM MTSET and subsequently with 1.5 mM MTS-PEG 5000, Line c: proteoliposomes were incubated with 1.5 mM MTS-PEG 5000. Error bars indicate the standard deviation of the mean of 3 independent sets of experiments.

As an alternative to MTSET activation of the cysteine mutants, lysophosphatidylcholine (LPC) was used (26). Asymmetric insertion of LPC into the membrane bilayer activates mechanosensitive channels (27). As shown in Figure 4B and D, all the cysteine mutants together with the ones that were not activated by MTSET ($V15C^{Tb\text{-}MscI}$, $V22C^{Tb\text{-}MscI}$, and $I23C^{Tb\text{-}MscI}$) could be activated by LPC. The rate of calcein release upon addition of LPC was similar for all the mutants (Table 6). Together with the mobility shift assay, these results reinforce the observation that $V22^{Tb\text{-}MscI}$ and $I23^{Tb\text{-}MscI}$ are accessible to MTSET labeling, but the modification does not open the channel.

**Three LlMscL cysteine mutants in giant spheroplasts can be activated by tension in patch clamp**

To characterize MscL channels from *M. tuberculosis* and *L. lactis* at the single molecule level, we performed patch clamp electrophysiology using giant *E. coli* PB104 spheroplasts. For each mutant, multiple measurements were performed from at least three independent spheroplast preparations. The tension sensitivity of the channels was described as defined before (21) by the amount of pressure that is required to activate MscL relative to that of MscS.

Out of more than twenty independent patches, $WT^{Tb\text{-}MscI}$ could be activated in only three of them (Figure 5). None of the cysteine mutants Tb-MscI($V15C^{Tb\text{-}MscI}$, $A20C^{Tb\text{-}MscI}$, $V21C^{Tb\text{-}MscI}$, $V22C^{Tb\text{-}MscI}$ and $I23C^{Tb\text{-}MscI}$) could be activated in the patch clamp assay. As previously reported (8,28), the tension requirement to activate $WT^{Tb\text{-}MscI}$ in *E. coli* giant spheroplasts may be even higher in the cysteine mutants. This is in good agreement with our growth assay, since $WT^{Tb\text{-}MscI}$ did not show any GOF phenotype as compared to LlMscL and Ec-MscI.

**Table 7.** Electrophysiological analysis of LlMscL WT and its cysteine mutants.

| MscL type | MscL/MscS pressure ratio ± S.D. | Open dwell time (ms) | |
|---|---|---|---|
| | | τ1 | τ2 |
| $WT^{LlMscL}$ | 1.86 ± 0.1# | <1 | 3.9 ± 1.1 |
| $L15C^{LlMscL}$ | 1.67 ± 0.3 | <1 | 2.8 ± 1.1 |
| $G20C^{LlMscL}$ | Very high | ND | ND |
| $V21C^{LlMscL}$ | Very high | ND | ND |
| $I22C^{LlMscL}$ | 1.84 ± 0.3 | <1 | 21.8 ± 2.5* |
| $I23C^{LlMscL}$ | 1.62 ± 0.1* | <1 | 1.7 ± 0.7* |

# number of independent patches ≥ 4 * $p < 0.05$

Among Ll-MscL channels only the $WT^{LlMscL}$ and three cysteine mutants ($L15C^{LlMscL}$, $I22C^{LlMscL}$ and $I23C^{LlMscL}$) could be activated by tension in the patch clamp setup. The tension sensitivity of the $WT^{LlMscL}$ was 1.86 ± 0.1 (n=18). The tension sensitivity required to activate $L15C^{LlMscL}$ and $I22C^{LlMscL}$ was comparable to that of $WT^{LlMscL}$ with tension ratios of 1.67 ± 0.3 (n= 6) and 1.84 ± 0.3 (n=4), respectively (Table 7). Although $I22C^{LlMscL}$ could be activated by tension, only a few channels were open and mostly in subconducting state as compared to $WT^{LlMscL}$. The channel mostly visited the subconducting states rather than the full open state. The cysteine mutant, $I23C^{LlMscL}$, could be activated easier than the WT protein, with a tension sensitivity of 1.62 ± 0.1 (n=10) (Table 7). The channel activity of $I23C^{LlMscL}$ was comparable to that of wild-type LlMscL in terms of multiple channels being open. $G20C^{LlMscL}$ and $V21C^{LlMscL}$ expressed in *E. coli* giant spheroplasts could not be activated by applying tension in patch clamp. The conversion to a cysteine residue at these two positions reduced the tension sensitivity very much. Indeed, previous

studies by Folgering *et al.* (19) reported a very high MscL to MscS pressure ratio of 2.6 ± 0.3 and 3.7 ± 0.2. for G20C$^{LlMscL}$ and V21C$^{LlMscL}$, respectively. The observed tension requirement for G20C$^{LlMscL}$ and V21C$^{LlMscL}$ was expected since these two positions are close to the constriction point of the hydrophobic gate region. We could also analyze the kinetics of channel openings. The data show that the channels that could be activated (WTLlMscL, L15C$^{LlMscL}$, I22C$^{LlMscL}$ and I23C$^{LlMscL}$) presented a very flickering gating behavior. The dwell time analysis revealed two distinct time constants. In all tested mutants, the first time constant was shorter than 1ms. The second dwell time ranged from 2 ms to 4 ms (Table 7) for WT, L15C and I23C. However, I22C had the longest dwell time (21.8 ms) among all the mutants. This mutant behaves as an intermediate between non-activated G20C$^{LlMscL}$ and V21C$^{LlMscL}$ and activated mutant I23C$^{LlMscL}$.

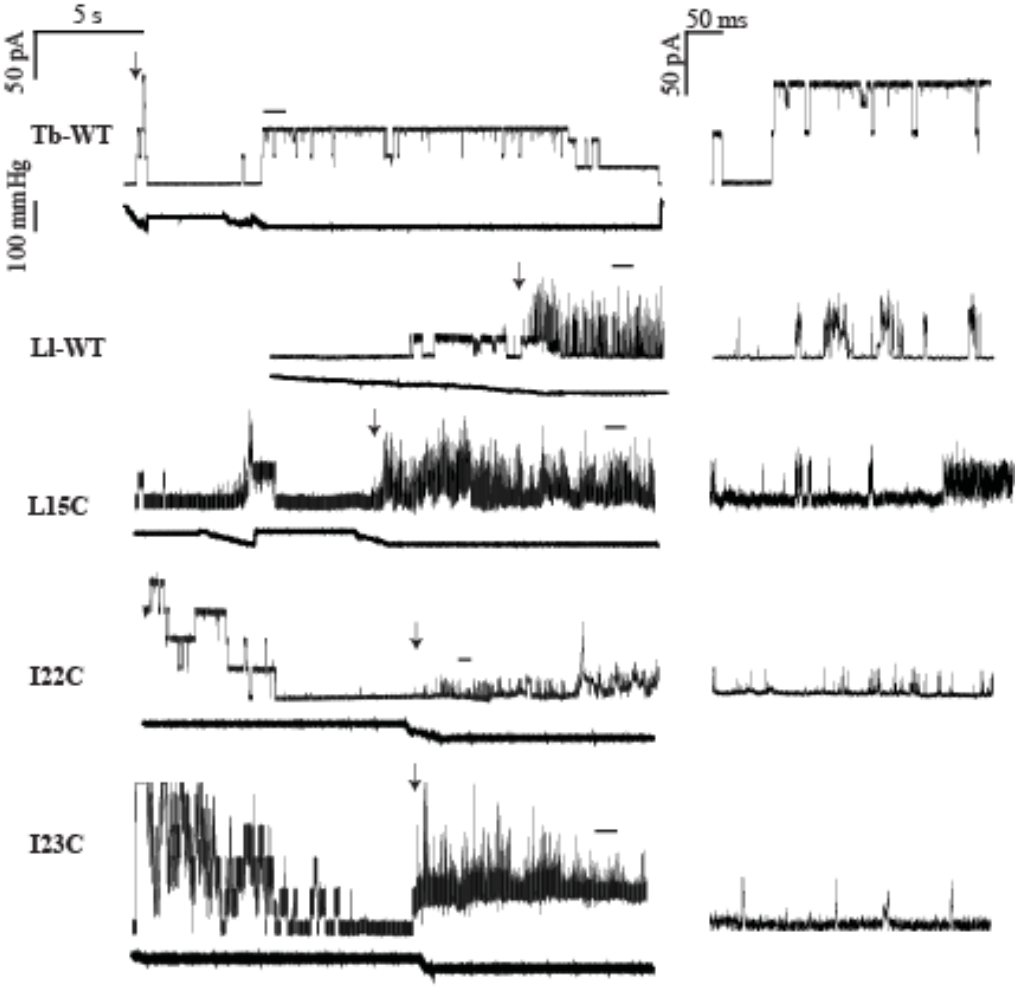


**Figure. 5** Patch clamp measurements of Tb- and Ll-MscL. Channel activities were recorded in excised patches from *E. coli* spheroplasts at -20 mV, using symmetric buffer conditions. Left panel: Full opening of MscL channels with applied negative pressure to the membrane. The WT LlMscL together with L15C, I22C and I23C could be activated as shown above. In the case of Tb-Mscl only the WT protein was activated. The asterics mark the opening of the mechanosensitive channel of small conductance (MscS) channels, which serves as an internal control for tension sensitivity; arrows mark MscL channels; line above MscL channels mark positions magnified in the right panel. Right panel: Detailed events of channel gating.

**Discussion**

In the current study, we have determined which pore residues of MscL from *M. tuberculosis* are functionally important. We have shown that given residues can be manipulated to open the channel in the absence of tension upon modification by MTSET. We compared the properties of the hydrophobic gate region residues from Tb-MscL (V15, A20, V21, V22, I23) to those of MscL from *L. lactis* (L15, G20, V21, I22 and I23) and *E. coli* (X17C, G22C, V23C, I24C, I25C). Individual residues of the Tb-MscL and Ll-MscL pore were mutated into a cysteine and were studied

both *in vivo* and *in vitro*. Furthermore, by heterologously expressing these channels both in *L. lactis* and *E. coli*, we investigated the *in vivo* membrane-dependence of channel activity.

In Tb-Mscl, A20 is the first residue around the channel pore that has been shown to be sensitive to the attachment of a charged chemical compound. In *E. coli*, the corresponding residue G22 is involved in hydrophobic gating of the channel (11-12). In *E. coli* MscL, changing G22 to any hydrophilic residue, or attaching a charge to this position via small chemical molecules, lead to channels that gate more easily and show a GOF phenotype (13). Previous mutagenesis studies of $A20^{Tb\text{-}Mscl}$ did not reveal channel activation in plate growth assays (10). We now show by labeling of XXX with MTSET that the modified channel opens in the absence of applied tension *in vivo*, as its *E. coli* partner G22C did. We also observed that this charge-sensitive activation of $A20C^{Tb\text{-}MscL}$ occurs when it is expressed in *E. coli* or reconstituted into synthetic lipid vesicles, but not when it is expressed in *L. lactis*. Surprisingly, when this channel was purified from *L. lactis* membranes and reconstituted into azolectin lipids, it could be activated by MTSET, suggesting that the conformation(al) (stability) of the protein might vary with the lipid environments. Indeed, it becomes more evident that the structure and function of membrane proteins depend on the lipid environment (29-35). In the case of Tb-MscL, it has been shown that, for the tension-induced activity of Tb-MscL, the anionic lipid phosphatidylinositol (PI) is important (35). Recently, in an ion mobility mass spectroscopy study, it was shown that lipids bind non-selectively and with high affinity to Tb-MscL and make the protein more stable (36).

The other charge-sensitive residue on Tb-MscL was V21, which also forms part of the pore constriction of the channel (7). Similarly, the pore residues of *L. lactis* $G20C^{LlMscL}$ and $V21C^{LlMscL}$ are responsive to charge-induced activation both *in vivo* and *in vitro*. Together, these results indicate that residues A20C and V21C in *M. tuberculosis* MscL, and the corresponding G20C and V21C in *L. lactis*, play a similar function in channel gating as does G22C in *E. coli* MscL.

In conclusion, we show that similar to *E. coli* MscL, hydrophobic gating is the gating mechanism adopted by *M. tuberculosis* and *L. lactis* MscL. We identified A20 and V21 in Tb-MscL and G20 and V21 in Ll-MscL as critical residues for channel gating. We also show that MscL channels might adopt slightly different conformations or conformational stability when expressed in different lipid environments.

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